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THE GROWTH AND TOXIN PRODUCTION OF *BACILLUS DIPHTHERIAE* UPON PROTEID-FREE MEDIA.

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OF all the problems which fall within the bounds of bacteriology today, one of the most important is that which concerns the production of toxins by certain pathogenic micro-organisms. The toxin of diphtheria has, no doubt, so far as its chemical nature and conditions of formation are concerned, received the greatest amount of study from investigators. It presents, moreover, a problem in the biochemistry of bacteria, than which, to the present time, no other has yielded more satisfactory results, considered both in their relation to pure science and to their practical application in the treatment and prevention of disease. And yet there are many points in regard to the ultimate nature of these toxic products, their relation to and interaction with the antitoxins, which, to say the least, are very far from being well understood. Furthermore, it is probable that these elements of the problem must remain a matter of speculation only, until a more adequate knowledge shall have been gained regarding the chemical nature of these toxic products, and the conditions which are either favorable or necessary to their formation.

Although, in the qualitative analysis of the toxic substance produced by *B. diphtheriae*, the investigator can recognize certain organic or inorganic bodies, he is still in the dark with reference to the ultimate proteid body, whose exact constitution he wishes to determine. It is difficult to determine whether the albumoses are the toxic products, or whether the true toxin may not, in some way, be associated with them. Some investigators assume that the toxin itself may be an enzyme which has a proteolytic action and produces the virulent, but secondary, toxic products in the body of the animal. Whatever answer may be given to these diverse and often contradictory views, the fact remains that the diphtheria organism, growing upon a proteid-free medium, can synthesize a toxic substance of a proteid nature; and that the toxin is probably not (as previously claimed by Brieger and Fraenkel) a toxalbumen resulting from the splitting up of proteid bodies within the medium.

Among the first to determine that the toxin of *B. diphtheriae* could be formed in a proteid-free medium was Uschinsky.[1] Since his discovery, however, reports have appeared, from time to time, stating that success has not been met with in growing the diphtheria organism upon proteid-free media. In reply to some of these criticisms, Uschinsky says:[2]

> Ich gebe zu dass auch ich viele Misserfolge gehabt habe. Jetzt aber besitze ich eine Kultur, welche auf meine Flüssigkeit vorzüglich wächst, und weit mehr Toxin produziert, als es bei mir früher der Fall war. Selbst 1½ c.cm. einer 4–6 Wochen alten filtrierten Kultur tödten sicher ein mittleres Meerschweinchen in 40–36 Stunden mit allen für Diphtherietoxin charakteristischen Erscheinungen.

Regarding the frequency of the growth upon his medium, Uschinsky continues:

> Die Ursachen meiner und anderer Misserfolge scheinen mir darin zu bestehen, dass wirklich nicht eine jede Diphtheriekultur zum Wachsen auf eiweissfreier Nahrlösung geeignet ist. Junge, frisch vom Menschen genommene Kulturen sind schwer auf dieser Lösung zu kultivieren; wogegen ältere, an saprophytische Lebensweise gewöhnte Kulturen leicht wachsen.

In the experiments about to be recorded, the writer has repeated Uschinsky's experiment, and has tried certain modifications of his medium together with some entirely new combinations. In all cases it was taken for granted that toxin-formations could not occur without at least a fair appearance of growth. With this fact in mind, the first aim of the writer was to obtain the best possible growth upon proteid-free media. Although in the first set of preliminary experiments the reaction of the media was adjusted to +0.45, as recommended by Hitchens,[3] in the later work (including all the combinations of media herewith presented), the reaction was made slightly alkaline, as determined by litmus.

USCHINSKY'S MEDIUM.

The first proteid-free medium to be tried was that recommended by Uschinsky. This was made up as follows:

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[1] Uschinsky, *Centralbl. f. Bakt.*, 1893, 14, p. 316.

[2] Uschinsky, *Centralbl. f. Bakt.*, 1897, 21, p. 146.

[3] Hitchens, *Jour. Med. Res.*, 1905, 13, p. 523.

Water	1,000	parts
Glycerin	30–40	"
Sodium chloride	6	"
Di-Potassium phosphate	2.5	"
Ammonium lactate	6.5	"
Magnesium sulphate	0.3	"
Calcium chloride	0.1	"
Asparagin	3.2	"

Upon this medium the writer attempted to grow pure cultures of the granular, the barred, and the solid-staining[1] varieties of *B. diphtheriae*, obtained fresh from the nose and throat of patients suffering from diphtheria in the Providence hospitals. Out of 70 pure cultures tried, only two gave a growth upon Uschinsky's proteid-free medium, after an incubation of five days or more. In these two instances, furthermore, the growth was very slight and limited to the solid-staining varieties of the organism.

It is also perhaps noteworthy that in these cases, where several tubes of proteid-free media were inoculated from the *same* blood-serum culture, usually *only one or two tubes* would manifest a growth, thus seeming to indicate that even out of a pure culture of *B. diphtheriae*, there might be only a relatively small number of individual organisms which would immediately take up growth upon Uschinsky's medium; and furthermore, that these, either by chance or by peculiar fitness, proved to be the solid-staining types of the organism.

NEW COMBINATIONS.

After this trial of Uschinsky's medium, the writer made use of several other combinations of proteid-free media, all of which contained asparagin for the nitrogen basis and varied somewhat, both qualitatively and quantitatively, in respect to the salt constituents. Although an immediate growth of fresh cultures of *B. diphtheriae* upon these media did not usually occur, still on some of them the growth was more frequent and slightly more profuse than upon Uschinsky's medium. Among the many new combinations tried, especially the following may be mentioned:

Asparagin	1.50	parts
Glycerin	2.50	"
Sodium chloride	0.10	"
Di-Potassium phosphate	0.10	"
Potassium nitrate	0.03	"
Ammonium lactate	0.08	"
Distilled water to make	100.00	"

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[1] Wesbrook's classification, *Trans. Assoc. Amer. Physicians*, 1900.

Upon this medium very few cultures of *B. diphtheriae* grew; in fact only two out of 20 grew at all, and these two were types of D^2 and E^2 (Wesbrook's classification).

In the following medium the percentage of glycerin and sodium chloride was increased, and calcium chloride and ammonium phosphate were added:

Asparagin	1.00 parts
Glycerin	4.00 "
Sodium chloride	0.60 "
Di-Potassium phosphate	0.03 "
Ammonium phosphate	1.75 "
Calcium chloride	0.01 "
Distilled water to make	100.00 "

In this medium six cultures grew slightly, but none very well. Of these six, three were D^2.

In the next medium ammonium lactate was omitted:

Asparagin	1.00 parts
Glycerin	4.00 "
Sodium chloride	0.60 "
Di-Potassium phosphate	0.30 "
Potassium nitrate	0.03 "
Calcium chloride	0.01 "
Ammonium phosphate	1.00 "
Distilled water to make	100.00 "

In this medium 14 cultures grew; one well, the others very slightly. Of the 14, eight were D^2, three were granular forms, and three barred. The one that grew most luxuriantly was a D^2.

Asparagin	1.00 parts
Glycerin	4.00 "
Di-Potassium phosphate	0.30 "
Magnesium sulphate	0.03 "
Potassium nitrate	0.03 "
Calcium chloride	0.01 "
Ammonium lactate	0.05 "
Ammonium phosphate	1.00 "
Distilled water to make	100.00 "

In the above medium, which, as will be noted, contains no sodium chloride, 27 cultures grew. Of these 27, seven grew fairly well, and 19 slightly. Of these 27, moreover, 12 were D^2, 6 were granular, and 9 were barred. The 2 which gave the most luxuriant growth were D^2.

The fifth lot of media had the following constitution:

Asparagin	1.00	parts
Glycerin	4.00	"
Sodium chloride	0.10	"
Di-Potassium phosphate	0.30	"
Magnesium sulphate	0.03	"
Ammonium lactate	0.50	"
Ammonium phosphate	1.00	"
Phosphate of iron	0.02	"
Distilled water to make	100.00	"

On the medium above mentioned, 18 cultures grew, most of them very slightly. Of these, 8 were D^2 or E,^{2} 7 were granular, 2 were barred and 1 was a mixed culture. Owing to an accident, however, the results of growth in this series of tubes were incomplete.

In all of the above combinations of proteid-free media tried, the only source of nitrogen was either the ammonium salts or the asparagin. Generally speaking, the results in the growth or the toxin production of *B. diphtheriae* upon these media were not pronounced. In fact the growth was not sufficient to warrant the use of these media as a basis for the deeper problem of determining what definite constituents of the media favored or prevented the formation of the toxic products. But few inoculations of these first attempts at growth were made. The principal reasons for this were, that in the majority of cases the growth was too slight to even hint that there could be even the slightest toxin formation; in the second place, before the growth had progressed a suitable length of time, the rapid evaporation of the water from the tubes in the incubator so concentrated the medium that it either prevented further growth of the cultures, or produced an uncertainty as to the exact proportion of the ingredients in the solutions. It was always noticeable, however, that the forms which grew most persistently, in spite of the changes in the density of the medium, were the solid-staining varieties. Of these last, several inoculations were made, but in only one instance did the result indicate a very high degree of toxin production.

On the theory that one reason for the slight growth in the different varieties of media to which allusion has been made, might be the difficulty in assimilating the nitrogen from the ammonium compounds or from the asparagin, it was decided to make use of some other simple nitrogen-containing compounds, as glycocol or urea.

In general, the results obtained from the employment of glycocol were far more encouraging than any results obtained through the use of asparagin. Urea, however, as also determined by Sullivan[1] in his work on the biochemistry of color-production by bacteria, was of little value, either in aiding the luxuriance of growth or for increasing the formation of toxin.

The first medium in which glycocol furnished the nitrogen basis was made up as follows:

Glycerin	3.0	parts
Sodium chloride	0.60	"
Calcium chloride	0.08	"
Magnesium sulphate	0.32	"
Di-Potassium phosphate	0.23	"
Ammonium lactate	0.75	"
Glycocol	0.10	"
Distilled water to make	100.00	"

Owing to the fact that several of the first few cultures with which this medium was inoculated grew without delay, a large number of cultures was not tried. The first two cultures which grew well had been on blood serum for a period of three weeks and were transferred to the proteid-free medium from a broth culture. Of these two, one was a culture of CC^1, the other of CC^2. Of a 16-day-old culture of CC^2 in the medium specified above, 5 c.cm. were inoculated into a guinea-pig weighing 533 grams. The pig died in 36 to 38 hours with the characteristic signs of diphtherial poisoning. The organism was recovered at the autopsy in the form of a D^2 culture.

In still another case, a culture of CC^2, having been grown for a period of 17 days in media of the same composition as the foregoing, killed a pig in 31 hours with typical diphtherial poisoning. In this case also, the organism was recovered in the form of a culture of C^2D^2. These results appear to indicate that glycocol, even in so small an amount as 0.1 per cent, is able to render possible, provided the other constituents of the medium are favorable, a good growth and a powerful toxin production of *B. diphtheriae* upon proteid-free media, even if the period of growth is no longer than 16 days. We recall that Uschinsky states that the period of growth of his cultures was from four to six weeks.

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[1] Sullivan, *Jour. Med. Res.*, 1905, 14, p. 109.

Another modification of the medium was made by substituting urea for the glycocol as the nitrogen basis.

Glycerin	15.00 parts
Sodium chloride	3.00 "
Calcium chloride	0.05 "
Magnesium sulphate	0.15 "
Di-Potassium phosphate	1.25 "
Ammonium lactate	3.20 "
Urea	1.60 "
Distilled water to make	500.00 "

In the above instance the growth was even slighter than when asparagin was used; and this was true for all the tubes inoculated. Consequently no inoculations from these cultures were made.

A further modification was attempted by combining urea and glycocol in the same medium, as follows:

Glycerin	17.00 parts
Sodium chloride	3.00 "
Calcium chloride.	0.04 "
Magnesium sulphate	0.16 "
Di-Potassium phosphate	1.25 "
Ammonium lactate	3.25 "
Glycocol	0.50 "
Urea	2.50 "
Distilled water to make	500.00 "

A 17-day-old culture of CC^1 upon this medium was inoculated into a guinea-pig and death resulted in 59 hours. In this case, at least, urea appeared to hinder rather than to favor either the luxuriance of growth or the degree of toxin production; for the same form of *B. diphtheriae*, grown for the same time and in the same medium, but without the urea, killed in from 36 to 38 hours.

TABLE 1.

COMPOSITION OF SOME OF THE MEDIA USED IN THE EXPERIMENTS.

	1	2	3	4
Am. Lactate.................	.65	0.08		0.05
Am. Phosphate...............			1.00	1.0
Asparagin	.32	1.5	1.0	1.0
Cal. chloride................	.01		0.01	0.01
Ferric phosphate..............				
Glycerin.....................	3 to 4	2.5	4.0	4.0
Glycocol				
Magnesium sulphate..........	.03			0.03
Potas. nitrate................		0.03	0.03	0.03
Di-Potas. phosphate...........	.25	0.1	0.3	0.3
Sodium chloride..............	0.6	0.1	0.6	
Urea.........................				

	CD	C^1D^1	C^2D^2	CD	C^1D^1	C^2D^3	CD	C^1D^1	C^2D^2	CD	C^1D^1	C^2D^2
Growth......................		S	S			S	S	S	G	S	S	G
Toxin production		O	O									

TABLE 1.—*Continued.*

	5			6			7			8		
Am. Lactate	0.5			0.75			3.2			3.25		
Am. Phosphate	1.0											
Asparagin	1.0											
Cal. chloride							0.05			0.04		
Ferric phosphate	0.02			0.08								
Glycerin	4.0			3.4			15.0			17.0		
Glycocol				0.1						0.5		
Magnesium sulphate	0.03			0.32			0.15			0.16		
Potas. nitrate												
Di-Potas. phosphate	0.3			0.23			1.25			1.25		
Sodium chloride	0.1			0.6			3.0			3.0		
Urea							1.6			2.5		
	CD	C^1D^1	C^2D^2	CD	C^1D^1	C^2D^2	CD	C^1D^1	C^2D^2	CD	C^1D^1	C^2D^2
Growth	S	S	S	L	L	L		S	L	S	S	L
Toxin production				H	H					M	M	

EXPLANATION.—In every case except Nos. 7 and 8 water was added to make 100 parts; in these to make 500 parts. S=slight, G=good, L=luxuriant growth; H=Inoculation resulted in death in less than forty-eight hours; M=Inoculation resulted in death in more than forty-eight hours.

It may be stated here that the three last-mentioned combinations of proteid-free medium were also tried, made up with agar in solid form. Although many other forms of bacteria developed luxuriantly upon these media, the growth of *B. diphtheriae* was slight. It was, however, in several instances sufficiently rapid to enable the writer to make a diagnosis of the diphtheria organisms after a period of 12 hours' growth.

STUDY OF ADAPTATION.

One other point of incidental interest was to determine the possibility of adapting to a proteid-free medium cultures of *B. diphtheriae* which, when fresh from the throat of man, would not grow at all on the proteid-free combinations. It is a well-known fact that many plants may, through a long and gradual process of adaptation, be fitted to live and grow in a new and materially different environment. In the present case the adaptation process was carried on as follows: To one part of broth were added portions of the proteid-free medium, in the following parts: 1, 2, 4, 6, 8, 10, 14, 18, 25, 30, 40, 50, 60, 80, 100, 140, 180, 210, 240, until finally the proteid-free medium was reached in its purity. In this system of adaptation, the tubes were inoculated successively, each from the tube preceding. A period of from 24 to 36 hours was allowed for the growth in each of the first tubes of the series, while for the tubes in the last of the series a longer period of time was found to be necessary.

By this process of adaptation five cultures of *B. diphtheriae*, of which two were of the D² type and the other three the CC¹ type, were brought successfully to the eighteenth series of adaptation tubes, in which the medium contained one part of broth to 210 parts of proteid-free medium. In every instance the solid-staining forms grew the most luxuriantly. In every instance, moreover, except in one culture of D², a strong toxin was developed, and the degree of toxicity appeared to be directly proportional to the age of the culture. After the cultures had once taken a hold, so to speak, any increment in the proteid-free basis appeared to make little difference in the strength of the toxin produced in the tubes beyond the fourth series (i. e., broth 1 part, proteid-free medium 6 parts). The records of the inoculations of the barred and the granular forms during the process of adaptation may be tabulated as follows:

TABLE 2.

Series	Age of Culture	Composition	Killed in
1........	3 weeks	Broth 1 pt.; Prot.-free 6 pts.	25 hours
2........	2 weeks	Broth 1 pt.; Prot.-free 80 pts.	32 hours
3........	3 weeks	Broth 1 pt.; Prot.-free 210 pts.	28 hours

These results seemed sufficiently definite to warrant the statement that certain forms of the diphtheria organism which will not grow normally upon a proteid-free medium can, by slow degrees, be fitted to such a life, and may be made to produce under such new life conditions a very strong toxin. There still remains a problem of interest to be solved in determining whether the adaptation results from a modification of the existing forms, or whether a selection of those varieties which are most suited to the new environment takes place. Regarding this question there are no data to be brought forward at this time.

PERIOD NECESSARY TO OBTAIN MAXIMUM TOXICITY.

There is a general unity in the opinion that the maximum toxicity for *B. diphtheriae* growing in broth is reached in from 40 to 50 hours after the inoculation. Uschinsky, on the other hand, ascertained that a period of from four to six weeks was required for the formation of a powerful toxin in his proteid-free media. In the tests which the writer made in this regard, it was quite apparent that the food materials in a proteid-free medium were much more slowly assimilated than in the case of a broth culture. In the majority of cases at least a day was necessary before the growth was at all noticeable; and the growth was seldom heavy before three to five days had elapsed. A number of instances appeared to indicate that the degree of toxicity, at least within certain limits, was directly proportional to the time that the culture had been in incubation.

RELATION OF THE DEGREE OF TOXICITY TO THE FORM OF THE ORGANISM AND TO THE LUXURIANCE OF GROWTH.

There can be but little doubt that the granular forms, so called, of *B. diphtheriae* are, as a rule, the most virulent. The solid-staining varieties, on the other hand, appear to be non-pathogenic in the greater number of cases. And yet the results of the writer's observations permit of little doubt that a single morphological variety of the diphtheria organism is decidedly modifiable; and that not only may the granular types of the organism be resolved into the solid-staining forms, but that the opposite may also be true. Furthermore, it is clear that the solid-staining types may, either with a maintenance of their original form, or with a change of that form within the body of the animal, prove highly pathogenic for guinea-pigs. The observations in this regard, were only incidental to the main problem in hand; but, in view of the prevalent difference of opinion upon this question, they may perhaps be appropriately mentioned at this time. On the first trial, eleven inoculations of pure cultures of D^2 were given to guinea-pigs before one culture proved to be pathogenic. The twelfth, a D^2 type, killed in 39 hours, with all the usual signs of diphtherial poisoning. When the organism was again recovered, it was found to be of the barred variety. On several other occasions also, when granular forms were inoculated, barred and solid-staining forms were recovered. In three successive cases where two cultures of CC^1 and one culture of C^1 C^2, grown for 17 days upon proteid-free media, were inoculated into guinea-pigs, they killed in from 37 to 59 hours, with all the characteristic symptoms. Without an exception, when the organism was recovered from the bodies, the D^2 form was alone present. One of these recovered cultures of D^2, after 48 hours of incubation in broth, was inoculated into a guinea-pig and resulted in death in 37 hours. The organism was recovered in the form of a D^2.

These facts appear to indicate beyond a doubt that important changes may be brought about in the form and toxin production of individual bacilli in a single culture of the organism; and that these different forms are determined in a great measure by the nature of the environment. Whether this distinct modification occurs as a result of a morphological change in all the individuals during a few successive generations, or whether by a less rapid process of selection and elimination of those morphological varieties, or different strains of the same morphological variety, which are not readily adaptable, this we are not yet able to determine. It is not altogether improbable, however, that there may be in the life of the diphtheria organism what may be roughly called a series of adaptive forms, each one of which may be best suited to a circumscribed condition of environment, there to produce, as the case may be, color, toxin, or even definite changes in form.

TOXICITY AND LUXURIANCE OF GROWTH.

As to the relation between the degree of toxicity and the luxuriance of growth, the experience of the writer would not warrant the drawing of any far-reaching conclusions. It is true, however, that the solid-staining types, whether they be true *B. diphtheriae*, pathogenic pseudo-diphtheria bacilli, or non-pathogenic pseudo-diphtheria bacilli grow more rapidly upon proteid-free media than do the granular types. Not only do the D^2 forms grow more rapidly, but the nature of their growth is far more luxuriant and of quite a different nature from that of the granular varieties. The typical growth for the granular type, on either broth or proteid-free media, is at the surface, where it forms a more or less delicate film. Upon shaking the tube, this film falls to the bottom as a fine precipitate or sediment. The medium from the surface to the bottom of the tube is, in the growth of the granular forms, nearly as clear as the contents of a sterile tube. It is the nature of the solid-staining types, on the other hand, instead of forming the surface growth and maintaining a clear mid-liquid, to produce in the medium a uniform cloudiness. This may, in time, settle to the bottom of the tube, but the medium never wholly loses its turbidity. This condition of growth was observable in all the series of cultures in proteid-free media as well as in the latter part of the series of adaptation tubes which have been mentioned above. In summation it may be said that, although a high degree of toxicity may often accompany a luxuriant growth, luxuriance of growth can never be used as a criterion of the degree of toxicity.

It may be of interest to mention one other point regarding the so-called involution-forms of *B. diphtheriae.* These, known by Wesbrook's classification as the "A" varieties, are generally conceded to represent forms which have become attenuated, both in virulence and in luxuriance of growth. This is indicated by the fact that when a culture of *B. diphtheriae* is grown upon a medium unfavorable to its growth, many of these forms develop, and they are often common in the throats of those who are recovering from an attack of diphtheria. In the writer's experience, these forms often developed, at the very last, in tubes of proteid-free media wherein the granular, and less frequently the solid-staining types, had found growth most difficult. These same involution-forms, in at least three cases, in our experiments, produced such a rapid and luxuriant growth in broth, that we were deceived by their macroscopic appearance into believing that these particular tubes were contaminated with some one of the large, pellicle-forming spore-bearers. By the examination of one tube, however, it was found that there was naught in the tube but a pure culture of the "A" varieties of *B. diphtheriae*, and that these composed, not only the heavy pellicle, which, in 24 hours, had become so dense that it restrained the liquid portion when the tube was inverted, but also the rapidly growing individuals beneath the pellicle. These individuals were the largest that have ever come under our observation, many of them measuring nearly ten micra in length, and under $\frac{1}{16}$-inch oil-immersion, rendering camera lucida drawings easily possible. It may also be noted that two of these cultures, referred to above, came originally from monstrous colonies growing upon blood-serum, one of which was 10 mm. in diameter. These were the only colonies of this nature ever seen by the writer.

SUMMARY.

1. *B. diphtheriae* will grow readily and luxuriantly in proteid-free media, and will produce therein as strong a toxin as in ordinary broth, though much more slowly.

2. Of the three nitrogen bases tried (asparagin, urea, and glycocol), urea seemed to be of slight value, while glycocol furnished the best growth and the strongest toxin. Asparagin appeared to give better results than urea, though it was not as satisfactory as was glycocol. There is no added value to be gained by using any two, or even three, of these compounds together.

3. Upon the following medium, cultures of *B. diphtheriae* were grown, and, after an incubation of 16 days, killed guinea-pigs in the course of 36 to 38 hours:

Glycerin	3.40	parts
Sodium chloride	0.60	"
Calcium chloride	0.08	"
Magnesium sulphate	0.32	"
Di-Potassium phosphate	0.23	"
Ammonium lactate	0.75	"
Glycocol	0.10	"
Distilled water to make	100.00	"

4. In several instances there were obtained cultures of pathogenic, solid-staining varieties of *B. diphtheriae* (C^2, D^2, E^2), which, upon inoculation, killed guinea-pigs in 37 to 60 hours.

5. Cultures of *B. diphtheriae* which will not at first grow upon proteid-free media, may be adapted to it by slow degrees; and whether during the process of adaptation, or after the adaptation, it is the solid-staining forms of the diphtheria organism which always manifest the most rapid and luxuriant growth.

6. A single morphological variety of *B. diphtheriae* is decidedly modifiable, and there are indications that there may be in the life of the diphtheria organism what may be roughly called a series of adaptive forms, each one of which is best suited to a certain condition of environment, where it may produce color, toxin, or change in form.